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A SPLIT HAND-SPLIT FOOT (SHFM3) GENE IS LOCATED AT 10Q24->25.

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The split hand-split foot (SHSF) malformation affects the central rays of the upper and lower limbs. It presents either as an isolated defect or in association with other skeletal or non-skeletal abnormalities. An autosomal SHSF locus (SHFM1) was previously mapped to 7q22.1. We report the mapping of a second autosomal SHSF locus to 10q24->25. A panel of families was tested with 17 marker loci mapped to the 10q24->25 region. Maximum lod scores of 3.73, 4.33 and 4.33 at a recombination fraction of zero were obtained for the loci D10S198, PAX2 and D10S1239, respectively. An 19 cM critical region could be defined by haplotype analysis and several genes with a potential role in limb morphogenesis are located in this region. Heterogeneity testing indicates the existence of at least one additional autosomal SHSF locus.

KEY WORDS: Split hand-split foot malformation, SHFM3 locus, mapping, chromosome 10, genetic heterogeneity.

INTRODUCTION

The split hand-split foot malformation (SHSF) is a dysostosis of the central digital rays which may occur sporadically or familially. In sporadic cases a single limb is usually involved while variable expression is frequently observed in families segregating the SHSF malformation. SHSF has been observed not only as an isolated defect (MIM 183600), but also in association with other skeletal (tibial aplasia/hypoplasia, MIM 119100) and non-skeletal defects such as the EEC (ectrodactyly, ectrodermal dysplasia, cleft palate) syndrome (MIM 129900), or ectrodactyly-cleft palate (ECP) syndrome (MIM 129830). Two clinical types of non-syndromic SHSF have been identified. Type I SHSF, characterized by the absence of tibial abnormalities and type II SHSF, characterized by the presence of tibial abnormalities. An autosomal dominant pattern of inheritance has been documented in both clinical types of familial non-syndromic SHSF [Zlotogora, 1995].

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Several reports have described individuals with SHSF who carry specific rearrangements involving regions 7q22.1, 6q16.3->q22.3, and 2q24->q31 [Tajar et al., 1989; Rivera et al., 1991; Braverman et al., 1993; Genuardi et al., 1993; Naritomi et al., 1993; Viljoen and Smart, 1993; Gurrieri et al., 1995]. These regions have been considered candidate areas harboring genes leading to SHSF. The 7q22.1 region is the one most *frequently* rearranged in individuals affected with SHSF and the EEC syndrome. The SHSF locus mapped in that region has been designated SHFM1 [Marinoni et al., 1994, Scherer et al., 1994]. Another SHSF locus on chromosome Xq26 was designated as SHFM2 [Faiyaz et al., 1993].

Fine physical mapping of the chromosomal breakpoints has defined a 700 kb critical region for SHFM1 [Scherer et al., 1994]. Segregation analysis in families with autosomal dominant SHSF using markers from the 7q22.1 critical region failed to identify genetic linkage between those markers and the SHSF phenotype [Gurrieri et al., 1994; Marinoni et al., 1994; Palmer et al., 1994]. A similar outcome was reached when families with SHSF were genotyped for markers localized on chromosomes 6q and 2q [Gurrieri et al., unpublished data].

Recently, the mouse dactylaplasia (*dac*) mutation [Chai, 1981] which appears to be analogous to SHSF, was mapped on mouse chromosome 19, in an area syntenic to human chromosome 10q24->25 [Johnson et al., 1995]. This observation prompted us to screen our panel of 8 SHSF families with markers from the 10q24->25 region. Four of the 8 families were found to be linked to several markers in the 10q24->25 area. Independently, another study also reported the localization of SHSF in the same chromosome region [Nunes et al., 1995]. Thus, the presence of a third SHSF locus (SHFM3) is established. Our data define a minimum critical region of 19 cM for SHFM3. Furthermore, the identification of unlinked SHSF pedigrees further expands the heterogeneity in SHSF by suggesting the existence of at least one additional autosomal locus.

MATERIALS AND METHODS

Families

Our family panel consisted of 8 multiplex families. The phenotypes of two families (families 2 and 6) were reported previously [Spranger and Schapera, 1988; Marinoni et al., 1994]. The presenting manifestation in all families was non-syndromic SHSF. In addition, a number of affected individuals in several families exhibited other defects (Table I).

Marker Genotyping

The panel of families was genotyped for the various markers in 3 laboratories. Two of the laboratories used radioactive labeling of the primers while the third laboratory used fluorescent labeling. For the radioactive labeling 40 to 100 ng of genomic DNA were subjected to 27 cycles of amplification in 1x PCR buffer (Pharmacia), 1.5 mM $MgCl_2$, 200 µM each dGTP, dCTP, dTTP, 50 µM dATP, 1 µCi [α-^{32}P]dATP, 1pmol each primer (Research Genetics) and 0.2 U of Tfl DNA polymerase (Epicentre technologies) in a total volume of 10 µl. Each amplification cycle consisted of denaturation at 94°C for 30 sec, annealing at 55°C for 75 sec, and elongation at 72°C for 15 sec. The

TABLE I: Clinical Findings in Families with SHSF Malformation Included in This Study

	1	2	3	4	5	6	7	8
Hypoplasia of forearm	-	-	-	-	-	+	-	-
Camptodactyly	+	-	-	-	+	-	+	-
Polydactyly	-	-	-	+	-	-	-	-
Syndactyly	-	-	-	+	+	+	+	+
Triphalangeal thumb	-	-	-	+	-	-	-	-
Hypoplasia/aplasia of lower limb	-	+	+	-	-	+	-	-

27 cycles of amplification were preceded by a 4 min 94°C denaturation step and followed by a 6 min 72°C elongation step. In each tube 20 µl of denaturation buffer (95% formamide, 0.5% bromophenol blue, 0.5% xylene cyanol) was added. Five µl of the amplified products were electrophoresed in a 6% polyacrylamide/7M urea gel for 2 hrs at 40 watts. Radiolabelled fragments were visualized by autoradiography after exposure on X-ray film, or on a phosphorimager (Molecular Dynamics). For the fluorescent labeling primers were purchased from Research Genetics with the forward primer of each pair tagged with either FAM or HEX. Primers for a CA repeat polymorphism in the PAX2 gene [Sanyanusin et al., 1995] were synthesized using a Beckman Oligo 1000 and the forward primer was labeled with FAM. PCR was performed in a total volume of 25 µl using 50 ng of DNA, 40 µM dNTPs, 0.5 µM of each primer and 1.2 µl of a Taq polymerase (Boehringer Mannheim), TaqStart Antibody (Clontech) mixture 100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl pH 8.3 according to Weissenbach et al. [1992]. The Taq polymerase-Taq Start Antibody mixture was prepared by adding 8.8 µl of dilution buffer (Clontech) to 2.2 µl Taq polymerase (5 U/µl) and 2.2 µl antibody as described by the manufacturer (Clontech). After completion of the reaction, FAM labeled products were diluted 1:5 and HEX labeled products were diluted 1:2. Products were analyzed on an ABI/Perkin Elmer Model 373A DNA Sequencer using GENESCAN software.

Linkage Analysis

Genetic linkage analysis was carried out using the LINKAGE program (V. 5.1) [Lathrop and Lalouel, 1984] with the DOLINK program [Curtis and Gurling, 1993]. Two-point analysis was performed using MLINK [Lathrop and Lalouel, 1984] and for multipoint analysis we utilized the FASTMAP program [Curtis and Gurling, 1993]. Autosomal dominant inheritance was assumed with a disease allele frequency of 0.00001. Lod scores were calculated under a full penetrance model (penetrance of 1.0) and a reduced penetrance model (penetrance of 0.7). Recombination frequencies were assumed to be equal for males and females. Heterogeneity tests were undertaken using the HOMOG program [Ott, 1983].

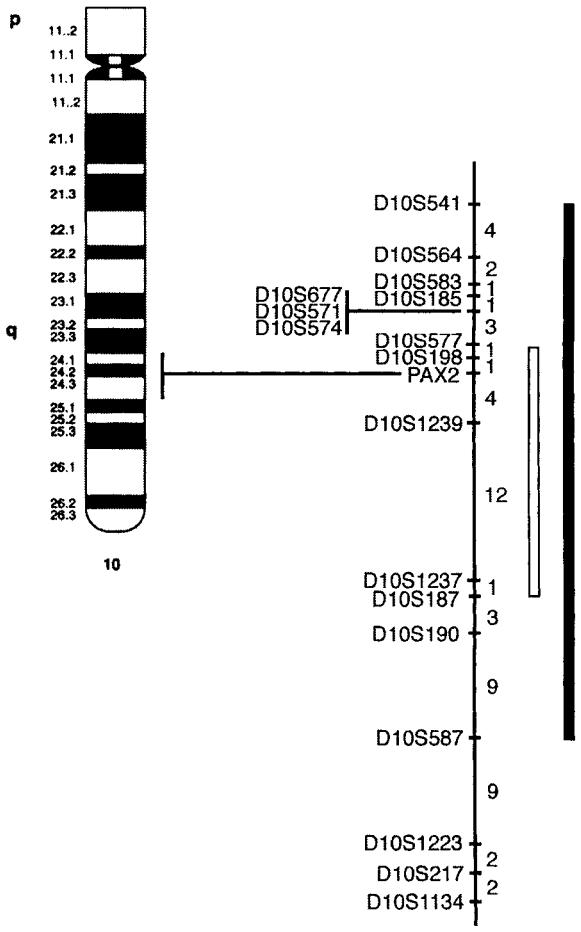

Figure 1: Genetic map of chromosome 10q24->25 loci. Open and solid vertical bars show the SHFM3 region defined by our data and that Reported by Nunes et al. [1995] respectively

RESULTS

Mapping and Localization of SHFM3

The family panel was genotyped with markers spanning from 10q24 to the telomere. In 4 families (families 1, 2, 3 and 6) numerous recombinants were scored and consistently negative lod scores were obtained (Table II). We concluded that these families were not linked to markers at 10q24->25. In the remaining 4 families evidence suggestive of genetic linkage was obtained initially with D10S677 (lod score of Z=2.6 at Θ=0.01, Table III). Formal heterogeneity testing using HOMOG provided evidence for 2 groups of families (χ^2=6.699, P=0.0048). A total of 17 markers located proximal and distal to D10S677 were used for linkage analysis (Fig. 1). By using a full penetrance model, markers D10S198, PAX2, and D10S1239 gave lod scores of Z=3.73, Z=4.33, and Z=4.33 at Θ=0.00 respectively (Table III).

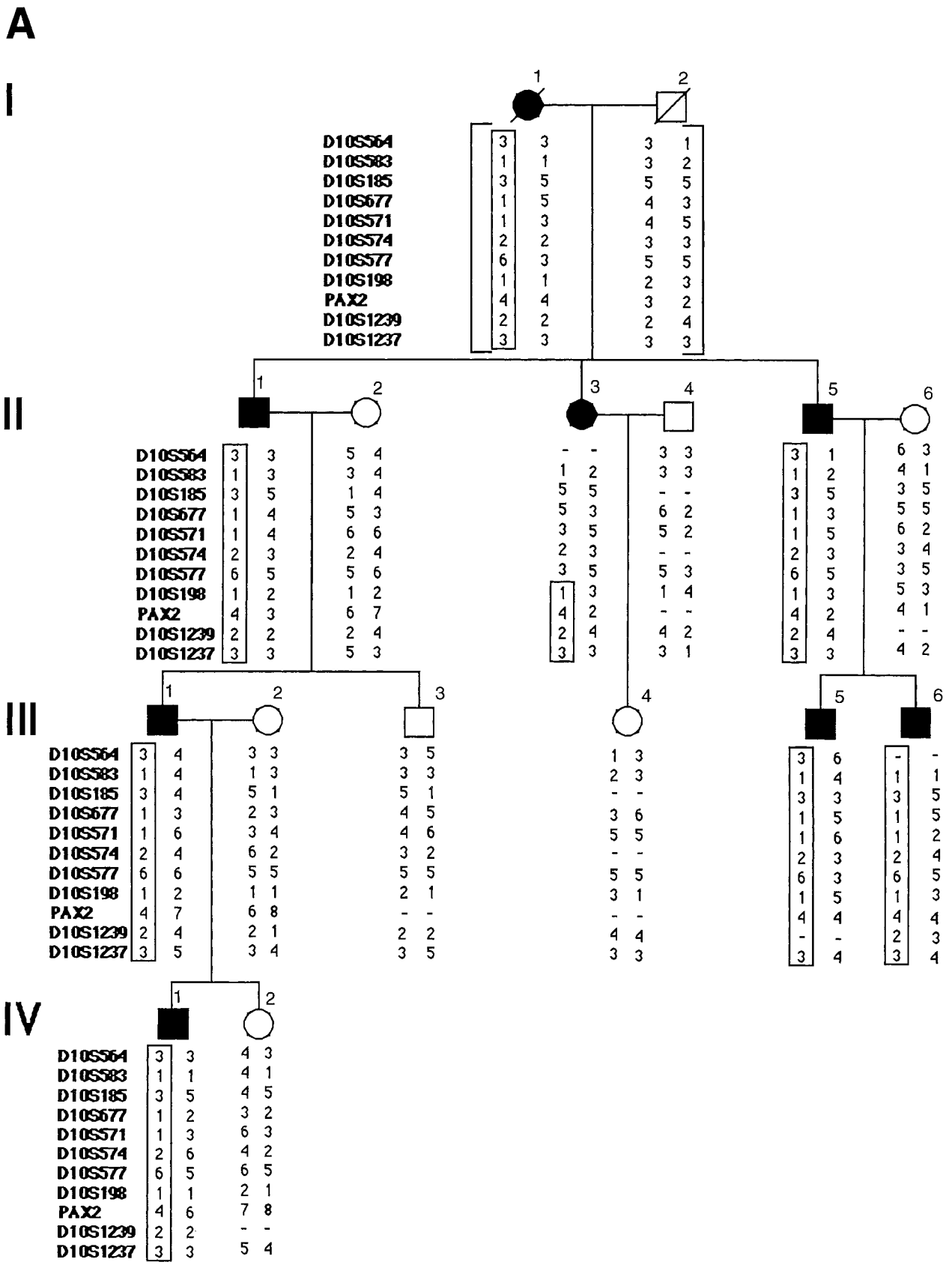

Figure 2a: Pedigree and 10q24->25 marker haplotypes for family 8.

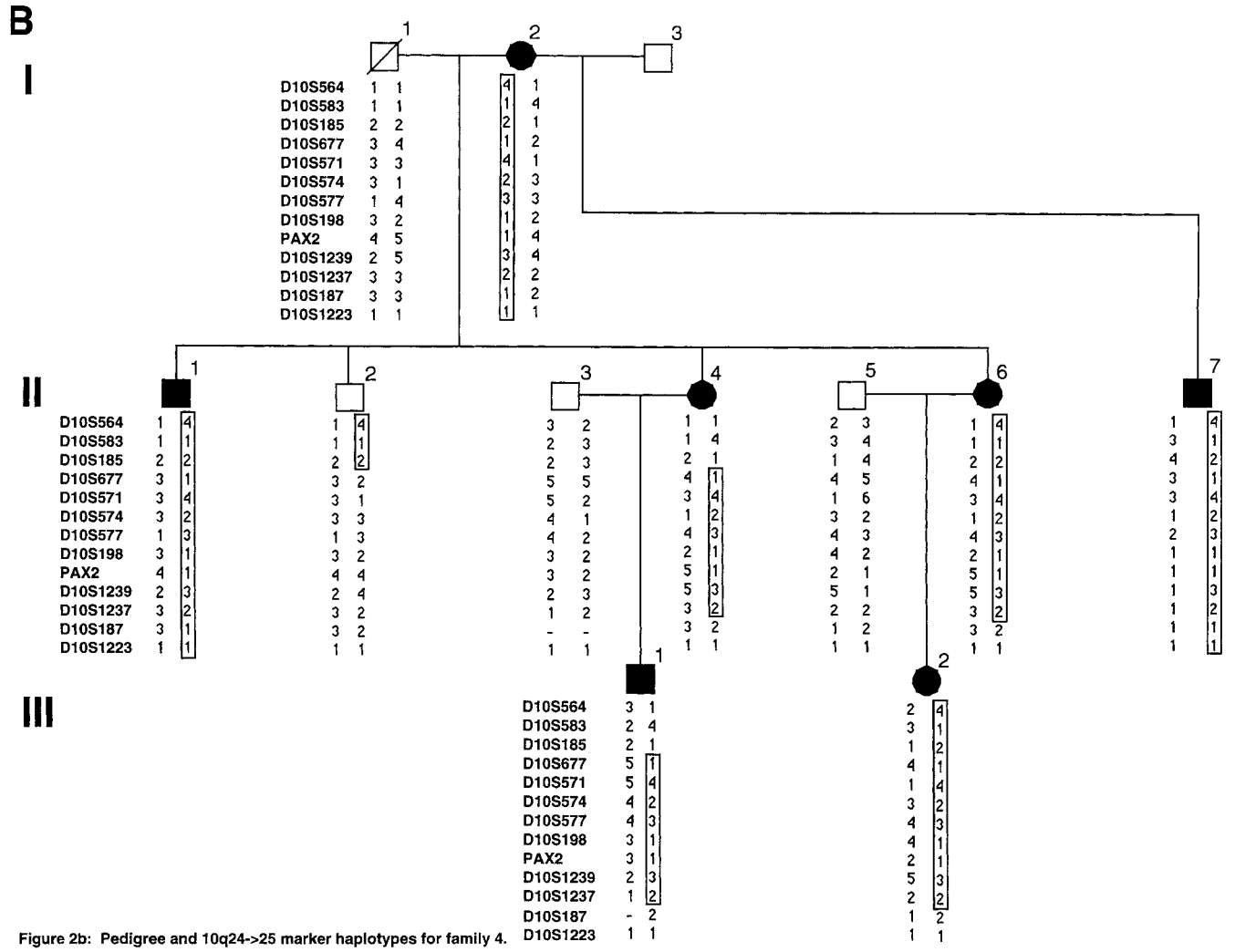

Figure 2b: Pedigree and 10q24->25 marker haplotypes for family 4.

TABLE II: Family-Specific Two-Point Lod Scores Between SHSF and D10S1239

	theta							
Family	0	0.001	0.01	0.05	0.1	0.2	0.3	0.4
1	-99.9	-4.66	-2.68	-1.23	-0.76	-0.25	-0.17	-0.02
2	-99.9	-3.97	-1.95	-0.64	-0.34	-0.16	-0.04	-0.01
3	-99.9	-10.0	-6.04	-3.36	-2.12	-1.18	-0.58	-0.24
4	1.80	1.80	1.78	1.65	1.49	1.13	0.73	0.31
5	0.60	0.60	0.59	0.54	0.47	0.32	0.17	0.05
6	-99.9	-11.0	-6.03	-2.76	-1.43	-0.48	-0.11	-0.01
7	0.60	0.60	0.59	0.55	0.50	0.40	0.29	0.15
8	1.33	1.32	1.29	1.18	1.03	0.72	0.43	0.19

Haplotype analysis documented a single cross-over between D10S577 and D10S198 in individual II-3 in family 8 (Fig. 2A), thus defining the centromeric boundary of the SHFM3 locus. Another cross-over between D10S1237 and D10S187 in individual III-4 in family 4 (Fig. 2B) defined the telomeric boundary. Under a reduced (70%) penetrance model we obtained lod scores greater than 3 with markers for the loci: D10S198, D10S574, PAX2, and D10S1239 (Table III). Multipoint analysis gave a maximum lod score of 5.33 between PAX2 and D10S1239 (Fig. 3).

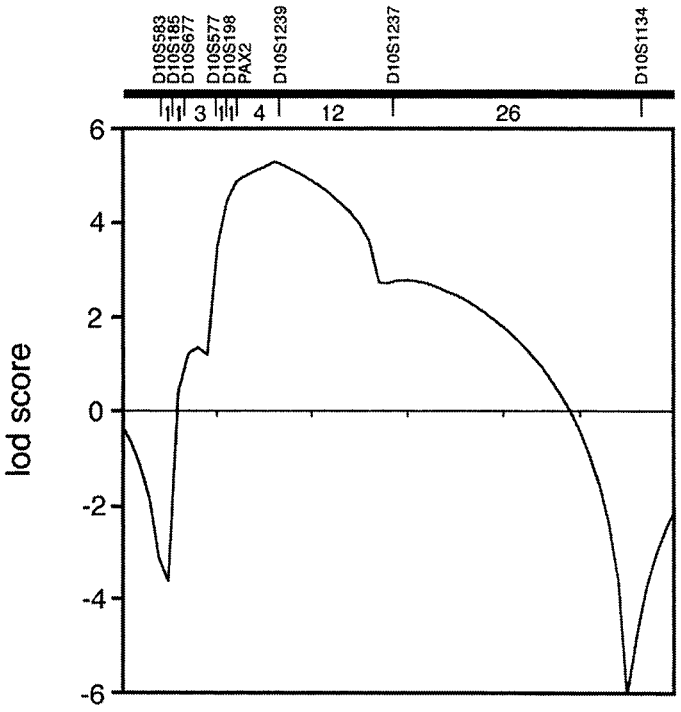

Figure 3: Location of SHFM3 defined by multipoint linkage analysis with nine 10q24->25 markers

DISCUSSION

The development of the limbs is a complex process characterized by the synchronous interaction of several intrinsic factors. Thus, it is not surprising that malformations in that developmental field might have several causes. The SHSF malformation is characterized by abnormalities of the middle rays of the hands and feet and frequently co-exists with tibial aplasia/hypoplasia. In this report we describe the genetic localization of SHFM3, a second autosomal locus underlying SHSF. Our data indicates that SHFM3 maps to chromosome 10q. Haplotype analysis defined a 19 cM interval between D10S577 and D10S187 for the SHFM3 locus (Fig. 1).

Independently, the observation of an unbalanced translocation with a 10q25.2 breakpoint in a patient with non-familial SHSF, hemimelia of the right lower limb, preaxial polydactyly, and cleft palate provided Nunes et al. [1995] with the stimulus to genotype an unrelated large SHSF family with markers from that region and establish genetic linkage. In this previous report a minimum and maximum critical region were proposed. Further genotyping of the family reported by Nunes et al. [1995] with markers localized in the region [Gurrieri et al., unpublished data] did not support a minimum critical region extending from D10S541 to D10S574 but it confirmed the maximum interval from D10S541 to D10S587 (Fig. 1). The latter interval overlaps with that defined by our studies. Thus, assuming the existence of a single SHSF locus at 10q24->25, our data reduce the previously reported critical region from 42 to 19 cM.

Two clinical types of familial non-syndromic SHSF have been proposed, one without (type I) and another with

TABLE III: Two-Point Lod Scores Between SHSF and 10q24->25 Loci Under Conditions of Full (i) and Reduced (ii) Penetrance

(i)	theta									
Locus	**0**	**0.001**	**0.01**	**0.05**	**0.1**	**0.2**	**0.3**	**0.4**	**Zmax**	**theta**
D10S564	-99.9	-3.89	-1.90	-0.55	-0.06	0.26	0.28	0.18	0.30	0.28
D10S583	-99.9	-4.07	-1.13	0.69	1.22	1.34	1.02	0.50	1.36	0.19
D10S185	-99.9	-7.83	-3.87	-1.27	-0.32	0.34	0.45	0.30	0.46	0.28
D10S677	-99.9	-0.59	1.34	2.42	2.60	2.28	1.60	0.74	2.61	0.09
D10S571	-99.9	-0.59	1.34	2.42	2.60	2.28	1.60	0.74	2.63	0.08
D10S574	-99.9	-1.04	0.89	1.97	2.16	1.89	1.30	0.61	2.18	0.09
D10S577	-99.9	-2.73	-0.77	0.45	0.82	0.92	0.71	0.35	0.94	0.19
D10S198	3.73	3.72	3.66	3.34	2.93	2.06	1.15	0.38	3.73	0.00
PAX2	4.33	4.32	4.25	3.90	3.45	2.51	1.56	0.67	4.33	0.00
D10S1239	4.33	4.32	4.25	3.92	3.49	2.57	1.62	0.70	4.33	0.00
D10S1237	-99.9	-0.74	0.23	0.77	0.88	0.76	0.50	0.22	0.88	0.10
D10S187	-99.9	-16.37	-9.41	-4.71	-2.84	-1.25	-0.56	-0.22	-0.22	0.4
D10S1223	-99.9	-8.77	-4.80	-2.15	-1.14	-0.34	-0.07	-0.01	-0.01	0.4
D10S1134	-99.9	-10.01	-5.07	-1.84	-0.68	0.11	0.25	0.14	0.26	0.32
(ii)	**theta**									
Locus	**0**	**0.001**	**0.01**	**0.05**	**0.1**	**0.2**	**0.3**	**0.4**	**Zmax**	**theta**
D10S564	-8.25	-3.74	-1.78	-0.51	-0.08	0.18	0.19	0.12	0.20	0.29
D10S583	-1.66	0.27	1.22	1.70	1.72	1.42	0.94	0.42	1.72	0.90
D10S185	-7.21	-3.43	-1.47	-0.20	0.24	0.48	0.43	0.25	0.50	0.22
D10S677	-0.65	1.27	2.21	2.64	2.58	2.10	1.41	0.63	2.65	0.07
D10S571	-0.69	1.27	2.21	2.63	2.57	2.09	1.40	0.62	2.64	0.07
D10S574	3.05	3.04	3.00	2.81	2.55	1.92	1.23	0.55	3.05	0.00
D10S577	-2.60	-0.64	0.33	0.89	1.00	0.90	0.64	0.30	1.01	0.11
D10S198	3.34	3.33	3.27	2.97	2.59	1.79	0.98	0.32	3.34	0.00
PAX2	3.94	3.93	3.86	3.53	3.11	2.24	1.36	0.57	3.94	0.00
D10S1239	3.83	3.82	3.75	3.44	3.05	2.22	1.37	0.59	3.83	0.00
D10S1237	1.40	1.39	1.38	1.27	1.13	0.83	0.51	0.22	1.40	0.00
D10S187	-7.76	-5.96	-4.92	-2.95	-1.82	-0.82	-0.39	-0.18	-0.18	0.40
D10S1223	-9.32	-5.11	-3.09	-1.61	-0.95	-0.38	-0.14	-0.05	-0.05	0.40
D10S1134	-13.08	-7.92	-4.13	-1.53	-0.58	0.08	0.20	0.12	0.22	0.31

tibial abnormalities (type II) [Marinoni et al., 1995; Zlotogora, 1995]. The previous classification most likely cannot be applied to individuals affected with SHSF and cytogenetic abnormalities (SHFM1). However, all 4 of our linked families could be classified as being SHSF type I. Similarly, the family reported by Nunes et al. [1995] was SHSF type I. In contrast, 3 of the 4 non-linked families in our panel were SHSF type II. The two types of SHSF frequently differ in the degree of observed penetrance with type I families usually exhibiting full penetrance while type II families often exhibit reduced penetrance lending further support to the notion of two familial SHSF types each with different clinical spectrum, pattern of inheritance, and etiology.

The SHFM3 locus has been localized to a region syntenic to mouse chromosome 19 where the mouse dactylaplasia (*Dac*) gene was recently mapped [Johnson et al., 1995]. Dactylaplasia phenotypically resembles SHSF type I. The expression of *Dac* is regulated by a modifier locus (*mdac*) as demonstrated by the existence of permissive and non-permissive strains of mice. It is tempting to speculate that a similar mechanism is operative in man. The *mdac* locus has also been mapped, on mouse chromosome 13 in an area syntenic to human chromosome 5q [Johnson et al., 1995].

Several genes have been mapped to the region of interest, including PAX2, HOX11, FGF8, FGFR2, HMX2, ZNF32, and RBP4 [Rocchi et al., 1989; Lu et al., 1992; Cannizzarro et al., 1993; Crossley and Martin 1995; Dressler et al., 1995; Rutland et al., 1995; Stadler et al., 1995]. PAX2 and HOX11 have been localized within a 3 cM region distal to D10S198 [Moschonas et al., 1996]. No recombinants with the intragenic marker of the PAX2 locus were observed in either our linked families or the family previously reported [Nunes et al., 1995]. However, PAX2 has been shown to be active primarily in embryonic hindbrain, neural tube and kidney rather than the developing limb bud [Dressler et al., 1995]. HOX11 appears to be involved in T-cell tumorigenesis rather than limb bud development as it is transcriptionally activated in translocations found in leukemia [Lu et al., 1992]. On the other hand, mouse hox11 is expressed in the branchial arches, neural crest cells and migrating mesoderm [Raju et al., 1993]. Thus, HOX11 cannot be ruled out as a candidate gene, especially since the association of gene mutations underlying both developmental abnormalities and leukemia has been documented for CBP in Rubinstein-Taybi syndrome and ANLL [Petrij et al., 1995]. The FGF8 gene is a likely candidate. In the developing mouse, FGF8 is expressed in several regions important for the outgrowth and patterning of the embryo, including the apical ectodermal ridge of the limb, the primitive streak, the branchial arches and nasal pits, and multiple areas of the central nervous system [Heikinheimo et al., 1994; Crossley and Martin, 1995]. In contrast, the FGFR2 gene is an unlikely candidate because of its localization between D10S190 and D10S217 which is distal to the SHFM3 region suggested by our data [Moschonas et al., 1996]. HMX2 is a homeobox whose expression pattern remains unknown [Stadler et al., 1995]. However, HMX2 is a potential candidate gene given the role of homeotic genes in pattern formation. ZNF32, a gene encoding a zinc-finger protein [Cannizzarro et al., 1993], is a possible candidate as transcription factors have been implicated in limb development as evidenced by the role of GLI3 in the Greig syndrome [Vortkamp et al., 1991]. RBP4, a gene encoding a retinol binding protein, has been localized to the candidate region [Rocchi et al., 1989] and, as retinoic acid derivatives play a role in normal limb development, cannot be excluded from consideration.

In conclusion, we have identified a split hand/split foot locus on chromosome 10q. This locus is associated with familial segregation of SHSF unlike the previously reported SHFM1. Additionally, the observation of families discordant for 10q, 7q, 6q, and 2q markers provides evidence for the existence of at least one more autosomal locus for this limb malformation.

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